See related Commentary on page vii

CDKN2A and *MC1R* Mutations in Patients with Sporadic Multiple Primary Melanoma

To the Editor:

The development of sporadic multiple primary melanoma (MPM) has been correlated with an underlying genetic susceptibility and/or exposure to UV light. To date, high-penetrance genes such as *CDKN2A* and *CDK4*, and low-penetrance genes such as *MC1R* have been shown to contribute to melanoma predisposition. The *CDKN2A* (or INK4A/ARF) locus at chromosome 9p21 encodes two alternatively spliced proteins, $p16^{INK4A}$ and $p14^{ARF}$, that demonstrate tumor suppressor activity and function as cell cycle inhibitors (Sharpless and Chin, 2003). *CDKN2A* mutations have been detected in 9%–15% of patients with MPM (Hayward, 2003); a family history of melanoma has been documented in many such cases. Germline mutations of *CDKN2A* affecting $p14^{ARF}$, however, have not been found in patients with sporadic MPM (Auroy *et al*, 2001). A low frequency of mutations of the *CDK4* oncogene has been shown in familial and sporadic melanoma (Goldstein *et al*, 2002), and no alterations have been detected in patients with MPM (Auroy *et al*, 2001). The *MC1R* gene encodes a seven-pass G-protein-coupled receptor that binds to the α-melanocyte-stimulating hormone causing a switch from red/yellow pheomelanin to brown/black eumelanin (Rees, 2000). Specific *MC1R* allelic variants (R142H, R151C, R160W, and D294H) have been associated with fair skin type and hair color (Valverde *et al*, 1996; Healy *et al*, 2000; Sturm, 2002) and an increased predisposition to melanoma development (Palmer *et al*, 2000; Kennedy *et al*, 2001).

In order to assess the presence of an underlying genetic susceptibility to the development of sporadic MPM, we screened 14 patients with MPM and no family history of the disease for germline and somatic *CDKN2A* mutations and for germline mutations in the $p14^{ARF}$-specific exon 1β of the *CDKN2A* gene, exon 2 of the *CDK4* gene and the entire coding region of *MC1R* gene.

The experiments conducted in this study were done in accordance with Helsinki principles and had the Ethical Committees' approval. Genomic DNA was isolated from peripheral blood using standard procedures. Tumor DNA and DNA from nontumorous tissue were extracted following microdissection from formalin-fixed, paraffin-embedded tissue sections as previously described (Peris *et al*, 1995). We used PCR amplification and DNA sequencing to screen genomic DNA and DNA isolated from tumor and nontumorous tissue samples for germline mutations in exons 1α, 1β, 2 and 3 of the *CDKN2A* gene, exon 2 of *CDK4*, and the entire coding region of *MC1R*. PCR primers were as previously reported for *CDKN2A* (Fargnoli *et al*, 1998) (GenBank accession numbers: U12818, U12819, U12820), $p14^{ARF}$-specific exon 1β (Mao *et al*, 1995) (GenBank accession number: L41934), and *CDK4* (Zuo *et al*, 1996) (GenBank accession number: U37022). PCR primers for the *MC1R* gene (GenBank accession number: AF153431) were the following: 1F-5′CAACGACTCCTTCCTGCTTC3′ 1R-5′-GTCACGATGCTGTGGTAGC3′; 2F-5′ACCTGCAGCTCCATGCTGTC3′; 2R-5′TGCCCAGCACACTTAAAGC3′, and PCR cycling conditions were: 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by a final extension step at 72°C for 7 min. Sequencing of the gel-purified PCR products was performed with the same primers used for PCR amplification and the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Automated sequencing was carried out with the ABI Prism 377 DNA sequencer.

To examine somatic alterations, loss of heterozygosity (LOH) and microsatellite instability (MSI) analyses at D9S974, D9S171, D9S126 were performed as previously reported (Peris *et al*, 1999). In cases in which LOH was found at 9p21, sequencing analysis of the *CDKN2A* gene was performed as described (Peris *et al*, 1999).

The study population consisted of 14 patients with MPM and no family history of melanoma (nine males and five females, with a mean age at the time of the first melanoma diagnosis of 46.9 y; range: 26–78 y). Phenotypic characteristics of the patients are reported in Table I. In eight patients, the melanomas were synchronous. In the remaining six patients, the diagnosis of the second melanoma was made between 1 and 8 y (mean: 4 y) after diagnosis of the first melanoma. Twenty-eight of 44 paraffin-embedded melanoma samples were available for analysis of somatic alterations.

Germline *CDKN2A* mutations were found in three of 14 (21.4%) MPM patients: two patients carried an ISV2+1G>A mutation whereas the third patient harbored the G101W mutation (Fig 1, Table I). No germline *CDKN2A* mutations were identified in exon 1β, encoding the $p14^{ARF}$ protein.

LOH at D9S974 was found in two of seven melanoma tissues of the patient (no. 14) who carried the G101W mutation. LOH at D9S171 in one allele and a 51_52insC at exon 1α in the second allele were detected in one of four melanomas from a patient (no. 11) who did not carry germline *CDKN2A* mutations (Fig 1, Table I). The 51_52insC mutation creates a frameshift and introduces a premature stop at codon 25, resulting in a truncated $p16^{INK4A}$ protein.

No mutations were detected in exon 2 of the *CDK4* gene in any of the 14 patients examined.

Abbreviation: MPM, multiple primary melanoma

Table I. Clinical characteristics of patients with MPM and results of germline and somatic *CDKN2A* mutations, and *MC1R* allelic variants

Clinical data of patients								Germline *CDKN2A* mutations		Somatic *CDKN2A* alterations				*MC1R* variants	
										Genetic instability					
Patient number	Patient origin	Sex	Skin type[a]	Hair color[b]	Eye color[c]	Number of melanomas	Age at the first diagnosis (y)	Mutation	Effect	D9S974	D9S171	D9S126	Mutations	Nucleotide change	Variant
1	Germany	F	II	Blond	Green	2	29	–	–	–	–	–	nd	g.178G>T	V60L
														g.451C>T	R151C
2	Germany	M	III	Blond	Blue	2	48	–	–	–	–	–	nd	g.274G>A	V92M
														g.478C>T	R160W
														g.942A>G	T314T
3	Germany	M	II	Red	green	6	38	IVS2+1G>A	Frameshift	–	–	–	nd	86_87insA	Frameshift
														g.451C>T	R151C
4	Germany	F	III	Light brown	Green	2	35	–	–	–	–	-	nd	g.451C>T	R151C
5	Germany	M	II	Light brown	green	3	67	–	–	–	–	–	nd	g.274G>A	V92M
6	Germany	F	II	Blond	Green	2	29	g.442C>T	Polymorphism	–	–	–	nd	–	–
7	Germany	M	II	Light brown	Blue	2	61	–	–	–	–	–	nd	g.274G>A	V92M
														g.942A>G	T314T
8	Germany	M	II	Red	Blue	5	48	–	–	–	–	–	nd	g.274G>A	V92M
														g.451C>T	R151C
														g.942A>G	T314T
9	Italy	F	II	Light brown	Light brown	2	70	IVS2+1G>A	Frameshift	–	–	–	nd	g.178G>T	V60L
														g.425G>A	R142H
10	Italy	F	II	Light brown	Green	3	26	–	–	–	–	–	nd	g.178G>T	V60L
														g.478C>T	R160W
11	Italy	M	II	Light brown	Green	4	64	–	–	NI	LOH	–	51_52insC	g.284C>T	T95M
														g.425G>A	R142H
12	Italy	M	III	Light brown	Light brown	2	78	–	–	–	–	–	nd	nd	nd
13	Italy	M	II	Light brown	Green	2	27	–	–	–	–	–	nd	g.478C>T	R160W
14	Austria	M	I	Red	Blue	7	37	g.301G>T	Mutation	LOH	–	NI	–	nd	nd

[a]Skin phototype according to Fitzpatrick's classification: I, always burns, never tans; II, always burns, tans lightly; III, seldom burns, tans well; and IV, never burns, tans deeply.
[b]Scalp hair color at age 18 was scored as: red, blond, light brown, dark brown, black.
[c]Eye color was classified as: blue, green, light brown, dark brown. – = no mutations; NI = not informative; LOH = loss of heterozygosity; nd = not done; MPM = multiple primary melanoma.

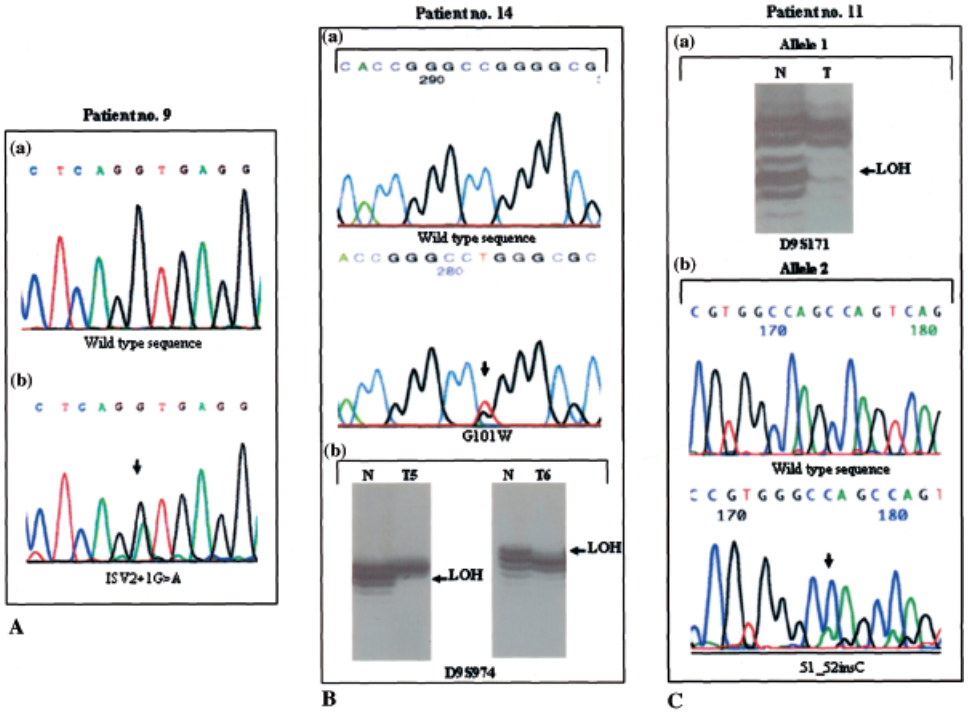

Figure 1
Germline and somatic CDKN2A mutations in patients with sporadic multiple primary melanoma (MPM). (*A*) Direct automated sequencing of germline *CDKN2A* in patient no. 9. (*a*) Wild-type DNA sequence and (*b*) G>A substitution at the splice donor site in exon 2. (*B*) Germline and somatic *CDKN2A* mutations in patient no. 14. (*a*) Germline wild-type DNA sequence (*top*) and C>T transition at base 301 in exon 2 (*bottom*); (*b*) loss of heterozygosity (LOH) at microsatellite marker D9S974, as indicated by arrow, was observed in melanomas T5 and T6 as compared with nontumorous tissue from the same patient; (*C*) Double somatic *CDKN2A* mutations in the absence of germline mutations in patient no. 11. (*a*) LOH at D9S171 (arrow) in Allele 1; (*b*) wild-type *CDKN2A* sequence (*top*) and a C insertion at nucleotide 51_52 (arrow) exon 1α in allele 2 (*bottom*).

Mutational analysis of *MC1R* gene was performed in 12 patients because DNA samples from the remaining two patients were no longer available. Seven *MC1R* allelic variants (V60L, V92M, T95M, R142H, R151C, R160W, and 86_87insA) were detected in 11 of 12 (91.7%) patients. Two patients (nos. 3 and 9) with germline *MC1R* mutations also carried the ISV2 + 1G > A *CDKN2A* mutation (Table I).

In a series of MPM patients with a negative family history of melanoma, MacKie *et al* (1998) identified *CDKN2A* alterations in two of the 17 patients screened, one of whom harbored the ISV2 + 1G > T mutation. Rutter *et al* (2003) recently demonstrated that this mutation reveals a cryptic splice site within exon 2 and also causes complete skipping of exon 2, resulting in the dual inactivation of $p16^{INK4A}$ and $p14^{ARF}$. In our study, a novel ISV2 + 1G > A *CDKN2A* mutation was detected in two of 14 patients with sporadic MPM. The effects of this mutation on splicing and on $p16^{INK4A}$ and $p14^{ARF}$ function needs to be characterized.

The third patient in our series with a germline *CDKN2A* mutation had G101W, which represents a well-known founder effect in melanoma families with a French, Italian, and American background (Ciotti *et al*, 2000). Recently, a common haplotype was also found in patients with sporadic MPM carrying the G101W mutation, further indicating that G101W is a common founder mutation (Auroy *et al*, 2001). In the absence of haplotype analysis in our patient and his relatives, we cannot exclude the possibility of this mutation representing a founder effect.

A single germline mutation (60ins16) in exon 1β of the *CDKN2A* gene has been detected in a patient with sporadic MPM (Rizos *et al*, 2001). In our study, no mutations affecting $p14^{ARF}$ were identified in patients with MPM, supporting the hypothesis that the $p14^{ARF}$ might play a pathogenetic role in only a minority of melanomas.

The lack of activating mutations at exon 2 of the *CDK4* gene, as detected in our series and in a previous report (Auroy *et al*, 2001), suggests that *CDK4* alterations may not be as prevalent in melanoma as originally thought.

Recent studies have demonstrated that specific *MC1R* variants act as modifier alleles, increasing the raw penetrance of *CDKN2A* mutations in Australian and Dutch melanoma kindreds (Box *et al*, 2001; van der Velden *et al*, 2001). We detected seven *MC1R* variants in 11 of 12 (91.7%) patients with sporadic MPM, which represents a much higher frequency as compared with that previously reported in other populations (Sturm *et al*, 2001). Three of the 11 MPM patients who carried *MC1R* allelic variants suffered the highest number of MPM and had red hair. Notably, two of these red heads were also *CDKN2A* mutation positive. Thus, our results might suggest another example of the effects of gene–gene interaction on disease risk.

In conclusion, we demonstrated that *CDKN2A* germline mutations may contribute to melanoma susceptibility in certain MPM patients and that *MC1R* allelic variants are frequent in such patients.

Ketty Peris,* Maria Concetta Fargnoli,* Alessia Pacifico,* Tiziana Surrenti,* Wilhem Stolz,† Peter Wolf,‡ Hans Peter Soyer,‡ and Sergio Chimenti§
*Department of Dermatology, University of L'Aquila, Italy; †Department of Dermatology, University of Regensburg, Germany; ‡Department of Dermatology, University of Graz, Austria; §Department of Dermatology, University of Rome Tor Vergata, Italy

The authors thank Barbara J. Rutledge, PhD for editing assistance.

DOI: 10.1111/j.0022-202X.2004.22532.x

Manuscript received November 11, 2003; revised January 13, 2004; accepted for publication January 17, 2004

Address correspondence to: Ketty Peris, MD, Department of Dermatology, University of L'Aquila, Via Vetoio – Coppito 2, 67100 L'Aquila, Italy. Email: peris@univaq.it

References

Auroy S, Avril MF, Chompret A, *et al*: Sporadic multiple primary melanoma cases: CDKN2A germline mutations with a founder effect. Genes Chromosomes Cancer 32:195–202, 2001

Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA, Hayward NK: MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet 69:765–773, 2001

Ciotti P, Struewing JP, Mantelli M, *et al*: A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families. Am J Hum Genet 67:311–319, 2000

Fargnoli MC, Chimenti S, Keller G, Soyer HP, Dal Pozzo V, Höfler H, Peris K: CDKN2a/p16^{INK4a} mutations and lack of p19^{ARF} involvement in familial melanoma kindreds. J Invest Dermatol 111:1202–1206, 1998

Goldstein AM, Chidambaram A, Halpern A, *et al*: Rarity of CDK4 germline mutations in familial melanoma. Melanoma Res 12:51–55, 2002

Hayward NK: Genetics of melanoma predisposition. Oncogene 22:3053–3062, 2003

Healy E, Flannagan N, Ray A, *et al*: Melanocortin-1-receptor gene and sun sensitivity in individuals without red hair. Lancet 355:1072–1073, 2000

Kennedy C, ter Huurne J, Berkhout M, *et al*: Melanocortin 1 receptor (MC1R) gene variants are associated with an increased risk for cutaneous melanoma which is largely independent of skin type and hair color. J Invest Dermatol 117:294–300, 2001

MacKie RM, Andrew N, Lanyon WG, Connor JM: CDKN2A germline mutations in UK patients with familial melanoma and multiple primary melanomas. J Invest Dermatol 111:269–272, 1998

Mao L, Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ, Sidransky D: A novel p16^{INK4a} transcript. Cancer Res 55:2995–2997, 1995

Palmer JS, Duffy DL, Box NF, *et al*: Melanocortin-1 receptor polymorphisms and risk of melanoma: Is the association explained solely by pigmentation phenotype? Am J Hum Genet 66:176–186, 2000

Peris K, Chimenti S, Fargnoli MC, Valeri P, Kerl H, Wolf P: UV fingerprint CDKN2a but no p14^{ARF} mutations in sporadic melanomas. J Invest Dermatol 112:825–826, 1999

Peris K, Keller G, Chimenti S, Amantea A, Kerl H, Höfler H: Microsatellite instability and loss of heterozygosity in melanoma. J Invest Dermatol 105:625–628, 1995

Rees JL: The Melanocortin 1 Receptor (MC1R): More than just red hair. Pigment Cell Res 13:135–140, 2000

Rizos H, Puig S, Badenas C, *et al*: A melanoma-associated germline mutation in exon 1β inactivates p14^{ARF}. Oncogene 20:5543–5547, 2001

Rutter JL, Goldstein AM, Dávila MR, Ticker MA, Struewing JP: *CDKN2A* point mutations D153spl(c.457G$>$T) and IVS2$+$1G$>$T result in aberrant splice products affecting both p16^{INK4A} and p14^{ARF}. Oncogene 22:4444–4448, 2003

Sharpless NE, Chin L: The INK4A/ARF locus and melanoma. Oncogene 22:3092–3098, 2003

Sturm RA: Skin colour and skin cancer – MC1R, the genetic link. Melanoma Res 12:405–416, 2002

Sturm RA, Teasdale RD, Box NF: Human pigmentation genes: Identification, structure and consequences of polymorphic variation. Gene 277:49–62, 2001

Valverde P, Healy E, Sikkink S, *et al*: The Asp84Glu variant of the melanocortin 1 receptor (MC1R) is associated with melanoma. Hum Mol Genet 5:1663–1666, 1996

van der Velden PA, Sandkuijl LA, Bergman W, Pavel S, van Mourik L, Frants RR, Gruis NA: Melanocortin-1 receptor variant R151C modifies melanoma risk in Dutch families with melanoma. Am J Hum Genet 69:774–779, 2001

Zuo L, Weger J, Yang Q, *et al*: Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet 12:97–99, 1996